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ON THE CULTIVATION OF TRYPANOSOMA BRUCEI.*

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IN our paper on the cultivation of *Trypanosoma lewisi*[1] we showed that this parasitic protozoön could be readily cultivated outside of the living body. At that time we expressed the belief that the same method would in all probability be applicable to other trypanosomes. This view has been confirmed apparently by Rabinowitsch and Kempner,[2] who, in a footnote, briefly state that the method is suitable for the cultivation of the trypanosome of Caderas. In a recent preliminary note[3] a brief summary was given of our work on *Trypanosoma brucei*, and the purpose of this paper is to present the details of that investigation.

Our knowledge of this interesting parasite dates back only to 1895, in which year Bruce published a preliminary report on the results of his study of Nagana, or the tsetse-fly disease of Zululand. A more extended report was published by him in

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[1] *Contributions to Medical Research*, dedicated to Victor Clarence Vaughan, Ann Arbor, Mich., 1903, George Wahr, p. 549.

[2] *Centralbl. f. Bacteriol.* (Orig.) 1903, 34, 816.

[3] *J. Am. Med. Assoc.*, November 21, 1903.

1897. It was clearly shown from these investigations that the disease was due to a flagellated protozoön similar to that previously described by Evans as existing in the Surra of India. This organism was subsequently designated by Plimmer and Bradford as *Trypanosoma brucei.* Bruce further demonstrated that the disease was spread by the bite of infected tsetse flies. The trypanosomes were found alive in the insects' proboscides up to forty-six hours after the flies had sucked infected blood. Motile parasites were still found in the flies' stomachs after 118 hours, but after 140 hours their stomachs were empty, and apparently dead parasites were found in the excreta. When contaminated flies were kept for twelve to forty-eight hours before they were allowed to bite healthy dogs, these only sickened on the thirty-second to thirty-eighth day, instead of after two weeks, as is usually the case. Nagana blood when removed aseptically produced the disease when kept for four days, but not after seven days. The blood when dried on threads produced infection after twenty-four hours in only one of three animals.

Kanthack, Durham, and Blandford in 1898 published the results of their studies on the trypanosome of Nagana. The material for this study was obtained from the blood of an infected dog brought to England from Zululand in 1896. We may say in passing that all of the subsequent investigations made in Europe, except that of Martini, were made with trypanosomes derived from this source. The observers mentioned found that in drawn blood, or in serous fluids, the hematozoa soon became motionless. This may occur rapidly, for instance in twenty minutes, but generally some motile specimens may be found after two to three days; sometimes, indeed, after as long as five or six days. Within a cadaver the blood and organs became non-infective in about twenty-four hours. They made numerous attempts at cultivation in normal blood, but while they observed the formation of tangles or agglutination masses, and, eventually, degeneration forms represented by masses of spherules, they failed to obtain any evidence of multiplication.

Plimmer and Bradford, in 1899, likewise noted the rapid disappearance of the trypanosomes from the blood after death, and in a measure ascribed this to the rapid onset of decomposition. While the blood in the dead body may lose its infectivity in a few hours after death, they found that a large quantity of blood (200 c.c.) kept in a sterile flask, in an atmosphere of oxygen, was infectious for at least three days. Under the cover-glass, desiccation being prevented by means of a paraffin ring, they were able to observe motile trypanosomes for six days after removal from the body. They found that an addition to the blood of one-tenth its volume of a 5 per cent. solution of sodium citrate served to prolong the vitality of the trypanosomes.

In their admirable study of the Nagana trypanosome, Laveran and Mesnil (1902) showed that a similar result is attained when the trypanosome blood is diluted with serum. In a mixture of equal volumes of defibrinated Nagana rat blood and horse serum the organisms were motile for three days, whereas they had disappeared from the undiluted blood in that time. They also noticed that the serum of refractory animals, such as birds and man, was as serviceable as that from susceptible animals.

On the other hand, human serum, unlike all other sera, was found to exert a specific action upon the trypanosomes of Nagana in infected mice and rats. The subcutaneous injection of $\frac{1}{2}$ to 1 c.c. of the human serum into these animals, at the time when the trypanosomes are just beginning to appear in the blood, causes the parasites to disappear in from twenty-four to thirty-six hours. The blood then remains free from the organisms for four to eight days, exceptionally for twelve, eighteen, or nineteen days, after which they reappear. On subsequent injection of serum they again disappear for a short time. In this way the life of the animal may be prolonged, as with injections of arsenite of soda, but eventually death results. In four instances, however, one or two injections of the serum in mice were followed by recovery. More recently (July, 1903) Laveran showed that human serum exerts a similar action upon the trypanosomes of Caderas and Surra.

Laveran and Mesnil studied the action of heat and cold upon the Nagana trypanosomes, and their results may be briefly summarized. Nagana blood which has been heated for three hours at 40° infects mice, but the period of incubation (five to six days) is a trifle longer than that observed when unheated blood is used. When the blood was heated for one hour and twenty minutes at 42°, the period of incubation was prolonged to eight days; that which had been heated at 44.5° was no longer infective. From this it is evident that a temperature of 40–42° kills the trypanosomes slowly, while that of 45° is rapidly fatal.

The statement was made in our first paper that Laveran and Mesnil found the trypanosomes alive after an exposure of one-half to two hours at – 50 to – 55°. The blood in question was first exposed for half an hour to – 15°, and then for five minutes to – 50 to – 55°, after which, on allowing it to thaw out for two hours, the trypanosomes were found to be motile and infectious. From a private communication of Dr. Mesnil we learn that he has found the Nagana and Caderas trypanosomes to resist the temperature of liquid air (– 191°) for a quarter of an hour.

Like the previous observers, Laveran and Mesnil found that Nagana blood when left *in vitro* may lose its virulence in forty-eight, or even in twenty-four hours. When mixed with physiological salt solution the trypanosome may be alive for three days, but the result is not constant. Even when the blood is kept in the ice-box at + 5 to 7° C., the virulence is lost in from three to five days, the same as when kept at the room temperature. It is worthy of note that they found such non-virulent blood to contain, at times, slightly motile trypanosomes. We will have occasion to show that living cultures of *Trypanosoma brucei* may be wholly non-virulent.

From the foregoing summary it will be noted that no one has been able to keep the *Trypanosoma brucei* alive for more than six days, and at no time has any evidence of multiplication *in vitro* been observed. Moreover, the virulence of the blood rapidly disappears, and only exceptionally is found to persist up to the fourth day.

The immediate purpose of our investigation was to demonstrate that the Nagana trypanosome, notwithstanding its apparently obligative parasitic character, could be cultivated *in vitro* as in the case of the rat trypanosome. We were able to undertake this work through the kindness of Dr. H. Wolferstan Thomas, late Fellow at McGill Medical College and now of the Liverpool School of Tropical Medicine, who with extreme cordiality supplied us with the infected blood. It gives us very great pleasure to thus acknowledge his valuable help, without which it would not have been possible to arrive at our results as early as we have. Our Nagana material is derived from that brought to England by Dr. Waghorn in 1896, and is therefore the same as that employed by the English and French investigators.

The methods which we have employed in this work are essentially the same as those described in our first paper, and for that reason need no detailed consideration at the present time. It may be stated that the sealing of culture tubes and flasks has been effected by means of thick rubber caps, which are more convenient than the sealing wax heretofore employed.

The culture medium usually employed in our work was ordinary nutrient agar containing variable amounts of defibrinated or laked rabbit blood. This medium by no means constitutes a perfect soil for the growth of *Trypanosoma brucei*, as will presently be seen, for the simple reason that we do not understand as yet the exact conditions which must be supplied in order to secure multiplication. A study of these factors is at present engaging our attention. Flask cultures are more successful than those in tubes, partly because of the thin layer of fluid, and partly because of the abundant supply of oxygen. The flasks were inoculated by means of capillary pipettes; the tubes usually with à platinum wire with 2 mm. loop.

The fact that this organism may appear in the blood of an animal in enormous numbers, and, within a few hours after death, may disappear entirely indicates an extreme sensitiveness to some as yet unknown substance which, presumably, forms in the blood as a result of post-mortem change. The living blood may be considered as a physiologically balanced salt solution with reference to the sensitive trypanosome. Upon the death of the blood this equilibrium is destroyed as a result of the cleavage changes which take place. The toxic action of the salts or ions thus formed must be neutralized before the trypanosomes can be made to grow. The decrease in the oxygen contents of the blood in the cadaver undoubtedly constitutes an additional factor in the destruction of trypanosomes.

It may be assumed that the blood remaining in the cadaver acquires a greater amount of the toxic compounds from the surrounding tissues than is the case when the blood is at once transferred to a tube. This, together with the fact that the oxygen contents are decreased, explains the more rapid death of the trypanosomes in the cadaver as compared with the test-tube. We have had no difficulty in keeping defibrinated Nagana rat blood in the test-tube, in the presence of oxygen for seven days.

The rat trypanosome is far less sensitive to the post-mortem alterations in the blood, and, for that reason, it may be kept alive in defibrinated blood for weeks and even for months, and, moreover, as will be seen, it will grow well on our culture medium when the Nagana parasite fails entirely.

The result of our first experiment, while not exactly successful, served to assure us that cultivation was possible. The blood of a white mouse, extremely rich in trypanosomes, was planted on a medium consisting of equal parts of defibrinated rabbit blood and ordinary agar. Examinations, made from day to day, showed that the organisms originally planted gradually decreased, till on the eighteenth day only one tube was found to contain a few motile trypanosomes. On this day the liquid contents of the tube were injected into a white rat, but no infection followed, showing that the organisms had become too enfeebled. Moreover, transplants made to fresh media failed to develop. The trypanosomes in the original tube showed more or less marked morphological alteration, appearing as spherical or kite-shaped forms, or as masses of small, round, granular bodies representing the débris of agglutination clumps or tangles, and only occasionally were typical organisms met with.

The significant feature of this experiment was the fact that the trypanosomes were kept alive for eighteen days, or three times longer than had been observed by others.

Notwithstanding numerous modifications of our technic, all our attempts to obtain cultures from the first twenty-five animals failed. In the inoculated tubes the trypanosomes would usually die out on about the fifth to the seventh day; exceptionally they were found to be still alive on the ninth, tenth, eleventh, fourteenth and eighteenth day. The trypanosomes which were placed in the incubator usually died out in two or three days.

The culture media were constantly checked by inoculating control tubes with *Trypanosoma lewisi*, which invariably gave an abundant growth. Clearly, the Nagana trypanosome required other conditions than did the rat parasite. This fact was brought out early, and in a most unexpected manner, as will be seen from the following:

Owing to a scarcity of new white rats, it became necessary to inoculate a rat which had previously been injected with *Tr. lewisi* but had entirely recovered. At least, repeated examinations of the blood failed to show any parasites. Four days after the inoculation with Nagana the rat died and the cadaver was placed on ice over night. In the morning, the blood, which showed about one organism in each field of the microscope, was planted into several tubes of blood agar.

In the course of a week excellent growths of a trypanosome were obtained, and at first it seemed as if the cultivation of *Trypanosoma brucei* was realized. In appearance the organisms corresponded exactly with the forms observed in cultures of *Trypanosoma lewisi*. This fact, together with the uniform failure at that time to duplicate this result from other Nagana animals, and the unsuccessful inoculation of mice and guinea pigs, soon convinced us that the culture obtained was one of *Tr. lewisi* and not of *Tr. brucei.*

This experiment was repeated with like result. A wild rat infected with *Tr. lewisi* was inoculated with Nagana. Both parasites appeared in the blood and could easily be recognized during life. After death the blood was transferred to culture tubes, and, as in the previous case, in the course of a week or two abundant growths of *Tr. lewisi* were obtained, but no sign of the Nagana parasite could be detected.

Apart from the differences in the morphological characteristics of the pure cultures of the two trypanosomes, which, as will be presently pointed out, are quite considerable, we have in the fact just given a ready means of differentiating the two organisms. A somewhat similar procedure was suggested by Koch,[4] who found that when the rat blood which contained the two trypanosomes was injected into a dog the latter died of Surra (Nagana?). It is evident that by combining these two methods pure cultures of these organisms *in vitro* can be obtained. Thus the blood showing double infection can be inoculated into blood agar (1:2) and also into a mouse, guinea pig, or dog. The blood-agar tubes will give a pure culture of *Tr. lewisi* since the Nagana trypanosome will not grow on this medium; while from the animal, after death, the *Tr. brucei* can be isolated by inoculating blood agar tubes (2:1).

From the second series of twenty-five Nagana rats, mice, and guinea pigs we succeeded in obtaining four positive results. These cultures or strains, obtained from four different animals, we will designate as A, B, C, and D.

CULTURE A.

Generation I.—This was started on August 27. The blood was drawn from the living heart of a white mouse, on the fifth day after inoculation, at which time it was literally swarming with the parasites. Six tubes of blood agar (2:3) were inoculated and set aside at the room temperature. As is usually the case, the trypanosomes originally planted gradually decreased in number. Involution forms, by which are meant spherical or kite-shaped individuals, motile or dead, as well as masses of small round bodies, the remnants of degenerated trypanosomes, were present.

On September 12 (sixteenth day), in only one tube out of the six, a few motile, typical cells were to be found, and that in a whole cover-glass preparation. Four days later several small masses composed of eight or ten actively motile cells were met with. The appearance and motion of these organisms were such as to justify the belief that they were not mere survivals of the original trypanosomes planted, but rather new individuals developed from these by multiplication. On September 18 (twenty-second day) there was no longer any doubt. The rosettes or colonies of actively motile trypanosomes had markedly increased in number and in size. The individual cells were vigorously active, and, in this respect, wholly unlike the slowly dying forms heretofore observed. The trypanosomes remained alive in this tube until after October 10, that is to say, for at least forty-four days.

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[4] *Reiseberichte*, 1898, 71.

Of the five tubes which were negative on September 12 one gave evidence of growth on the 16th. From that time the number of trypanosomes increased for about ten days, after which they began to die out, though some living cells were still present on the forty-fourth day.

Generation II.—This was made on September 18 from the first tube of the preceding generation. In order to insure sufficient inoculation the transplantation was made by means of a capillary tube pipette. Three of the six tubes of blood agar thus inoculated were kept at room temperature, and the other three were placed at 25°. Of the latter set one tube (1:1) showed a good, rich growth nine days later. Another of these tubes (3:1) on the same day showed some growth, but this was not as abundant as in the former probably on account of the large amount of water of condensation. Two tubes of the room set showed a good growth on October 8 (twenty days.)

Generation III.—This was made on September 28 from the rich 25° culture of the preceding generation. In this and subsequent inoculations the ordinary platinum loop was used. The blood agar employed was 1:1, and 2:1. Four tubes of each kind were inoculated. A set of two tubes, one of each kind, was set aside at room temperature. A like set was placed at 34°, and the remainder at 25°. Every one of these tubes gave a good growth. The 34° cultures developed rapidly, but died out shortly after the seventh day. One of the 25° cultures was alive on the twenty-fifth day, while one of the room cultures showed a few motile masses on the twenty-ninth day.

Generation IIIa.—This was inoculated on October 8 from one of the room cultures of Generation II. When examined on October 23, and again on October 27, many active single trypanosomes, as well as motile masses, were found. This material was injected on October 28 into four white mice (see Table I).

Generation IV.—This was made on October 2 from one of the 34° cultures mentioned above. The culture tube (2 : 1) was developed for nine days at 34°, and gave a very rich growth which persisted for thirteen days. A portion of this culture was injected on October 11, intraperitoneally, into a white mouse, with wholly negative result.

Generation IVa.—This was made on October 5 from one of the 25° cultures (1 : 1) of Generation III. Three tubes, 1 : 2, 1 : 1, and 2 : 1, were inoculated. These were developed at 25° for nine days, at which time the growth was abundant in the last two tubes.

A flask, 10 cm. in diameter, containing blood agar (2 : 1), was inoculated on October 6 and developed at 25°. It gave a very good growth. The contents of this flask were injected on October 14 into five white mice. The result of this injection will be referred to later. Another flask culture (IV*b*), inoculated on October 15 and kept at 25°, gave a good growth and was used on October 25 to inoculate flask culture V*b*. On November 6 a portion of the fluid was injected into two white rats (Nos. 9 and 10).

This same flask culture (IV*b*) served also on October 29 for starting another flask culture (V*c*).

A third flask of blood agar (3 : 2) was inoculated on October 15 from a 25° culture (1 : 1) of Generation III. On October 28 it showed a very fair growth.

Generation V.—This was made on October 11 from a 34° culture of Generation IV. The blood agar employed was 1:2 and 2:1, and the tubes after inoculation were kept at room temperature. Nine days later the first of these tubes showed many degenerations and only a few motile forms. The 2:1 blood agar showed no growth even on October 27. The failure to obtain a growth in this case may be due to the large amount of water of condensation present, or, what is more likely, the 34° culture from which the transplantation was made was in a too enfeebled condition, as indicated by its failure to infect a mouse.

Generation Va.—This was a flask culture (3:2) and was made on October 15 from the flask culture of October 6 mentioned under Generation IV*a*. The flask was kept at 25° for six days, at which time it was very rich in trypanosomes. After removing a portion of the fluid for injection into a rat and a mouse (see Table II), the flask was placed at 34° for five days. On October 26 it showed no motile forms, and only masses of round bodies were present. The higher temperature in this case clearly destroyed the culture. This material was then injected into two white rats (Table III). Generation V*b* was started on October 25 from one of the flask cultures of Generation IV. The medium (1:1) was contained in a flask, 10 cm. in diameter. For inoculation of animals with the culture see Table III.

Generation V*c* was also made as a flask culture (1:1) on October 29 from Generation IV*b*.

Generation VI.—A flask containing blood agar (1:1) was inoculated on October 23 from Generation V*a*, developed six days at 25°, and two days at 34°, and was set aside at 25°. Six days later trypanosomes were found, though in small number. On November 10 the culture was very rich. The slowness with which the growth came on is due probably to the exposure at 34°.

Generation VIa.—A flask culture (3:2) was made on November 12 from Generation V*c*. When left at 25° for four days it showed a fair growth; on the eleventh day it was very rich.

Generation VII.—A flask containing blood agar (1:1) was inoculated on November 10 from the preceding and placed at 25°. Actively growing trypanosomes were noted on November 16.

Generation VIIa.—A flask culture (3:2) was made November 16 from Generation VI*a*. In seven days at 25° it was quite rich in actively motile trypanosomes and was used to start Generation VIII*a*.

Generation VIII.—Three flask cultures were made on November 17 from Generation VII and set aside at 25° for six days, at which time a most excellent growth of trypanosomes was obtained. The flasks, however, developed mould contamination.

Generation VIIIa.—Two blood agar flasks were inoculated on November 23 from Generation VII*a*. On December 4 both flasks showed a very rich growth and served to start Generation IX. On December 5 another set of blood flasks was inoculated giving Generation IX*a*.

CULTURE B.

Generation I.—The blood of a white mouse, rich in trypanosomes, was drawn from the heart immediately after death and transferred to two blood agar tubes (1:1), which were then set aside at room temperature (September 7). Again, as in the first generation of Culture A, the original trypanosomes gradually disappeared so that on the ninth day only one individual could be detected under a whole cover glass. On September 29 (twenty-second day) many active rosettes were found and persisted up to October 8 (thirty-one days). Only one of the two tubes inoculated gave a growth.

A second generation made on September 29 showed a fairly rich growth on October 27. A third generation made on October 29 showed no organisms on November 6.

CULTURE C.

This culture is of interest, as it was obtained from a white mouse which was probably dead for some hours, since there were only about two trypanosomes present in each field of the microscope. It shows that, given the proper soil, even a few trypanosomes can be induced to grow on the artificial medium. The inoculation was made on September 10 into two tubes of blood agar (2:1), which were then placed at room temperature. Here again the organisms originally planted disappeared, so that when examined four days later nothing could be found. On September 29, the nineteenth day, one tube showed several small rosettes; on October 8 the growth was quite rich, and a few motile trypanosomes were still present on October 20 (fortieth day).

A second generation was made on September 29 on blood agar (1:1) and developed at 25°. On October 11 it showed a fairly rich growth which, when injected into a mouse, caused death in eight days.

Generation IIa.—On October 8 four tubes of blood agar (1:3, 1:1, 2:1, and 3:1) were inoculated and kept at room temperature. On October 27 the first of these showed no growth, while the other three were well developed, the last perhaps being the best.

Generation III.—A flask containing blood agar (1:1) was inoculated on October 27 and set aside at 25°. When examined on November 4 it showed many actively motile small rosettes.

CULTURE D.

The special interest which attaches to this culture is the fact that it was developed on a very weak laked-blood agar. On ordinary blood agar we have not been able to grow *Tr. brucei* when the amount of blood was less than 2:3. The blood was laked by adding 1 volume of distilled water to 3 of blood and keeping the mixture for several hours at 37°. Equal volumes of the laked blood and Thalmann's agar were used for the inoculation.

The blood, very rich in trypanosomes was drawn from the heart of a white mouse just before death and transferred to thirteen tubes (September 12). Hesse's, Thalmann's, and ordinary agar were used. Some of these agar tubes were mixed with defibrinated blood, while others were mixed with the laked blood. Of this set only two tubes developed a growth. One of these was the Thalmann laked-blood agar mixture; the other was on an ordinary blood agar (1:1). The cultures were planted on September 12, and when examined on the sixth, and again on the seventeenth day were wholly negative. On October 8 the laked-blood agar showed several active rosettes and contained active forms even on October 27 (45 days). On October 11, when twenty-nine days old, a portion of the fluid was injected into mouse No. 4 (see Table I), but no infection took place. On the same day transplantations were made to three blood agar tubes, but none of these showed any growth on November 6. The culture had evidently become too weak to grow or to infect a mouse.

The foregoing details furnish a sufficient demonstration of the fact that the *Trypanosoma brucei* can be cultivated outside of the living body. The cultures are, however, by no means as readily obtained as in the case of the rat trypanosome. We can safely say that the latter organism can be cultivated every time from an infected animal. This we have repeatedly done during the past six months without any failure. In every case the trypanosomes developed with certainty and in abundance.

The rat trypanosome finds the conditions suitable even when the amount of the blood in the medium is small. Thus it will grow on blood agar mixtures of 1:2, or 1:5, or even 1:10. The best conditions undoubtedly obtain with a 2:1 blood agar, on which the growth seems to be more rapid and more luxuriant.

The Nagana trypanosome, on the other hand, will only exceptionally grow on a medium which contains less than half its volume of blood. The best results seem to be given with 2:1 and even 3:1 blood agar mixtures. But even on these media the growth is by no means certain. Sometimes only one or two tubes develop out of a large number, although the medium is apparently the same in all of the tubes. This difficulty in causing the trypanosome to grow is best seen in the fact that we have succeeded in obtaining cultures from only four of the first fifty animals tested.

A noteworthy fact in connection with the starting of the initial cultures has already been alluded to. The original trypanosomes planted on the blood agar die out almost completely; so much so that an examination may fail to show any living forms, and thus give the impression that the culture is a failure and lead to the discarding of the tubes. And yet a few surviving organisms eventually succeed in adapting themselves to the changed condition, and from these the culture begins. The whole phenomenon is very suggestive of a survival of the fittest.

It has been shown that the cultures developed at the room temperature persist the longest. Several of these cultures contained living forms as late as the forty-fifth day. A temperature of 25° is well adapted for developing the cultures, but they do not last as long as those just mentioned. The trypanosomes may grow at 34°, but here again the cultures die out still earlier. A culture which has been grown at room temperature or at 25° when transferred to 34° dies out in a few days. It would seem from these facts as if an injurious substance persisted in the medium, and that its action was more marked the higher the temperature at which the growth took place. It is also possible that the waste products of the organism exert such an action.

The alteration which *Tr. brucei* undergoes in old cultures is brought out most clearly in their behavior to animals. It will presently be shown that, while the young cultures are almost as virulent as fresh Nagana blood, they become less and less virulent as they age, and, eventually, though living, may be wholly non-infectious.

INFECTION EXPERIMENTS.

It has already been pointed out that the virulence of blood from Nagana animals rapidly decreases, and that it disappears completely within three or four days after the removal of the blood from the animal. Living, motile trypanosomes may, however, be found in such material for five or six days. Our own experiments, made under different conditions, show: (1) that the original or surviving trypanosomes may retain their virulence for a greater length of time; (2) that virulent and non-virulent cultures may be obtained; and (3) that vaccination by means of non-virulent cultures is probable.

When trypanosome blood is transferred to blood agar tubes, the greater number of the trypanosomes which are thus carried over die out very rapidly, within two or three days, but some more hardy individuals survive for seven, ten, fourteen, or even eighteen days, without any apparent multiplication taking place. These survivals gradually lose their virulence, so that eventually no infection may follow the injection of motile trypanosomes. The similar behavior of blood, preserved in an ice-box for three days and showing slightly motile trypanosomes, has been noted by Laveran and Mesnil. In both instances the loss of virulence may be ascribed, with good reason, to the attenuation of the original surviving cells. On the other hand, it will be shown that pure living cultures may be obtained which are equally non-virulent.

Experiment 1. The liquid in a blood agar tube was inoculated with the blood of mouse No. 1, and was kept at room temperature. It showed a few motile trypanosomes on the eighteenth day (July 14) and was then injected into the peritoneal cavity of a white rat. No infection followed. On August 3, nineteen days later, the animal was inoculated with virulent Nagana blood. Infection resulted, the same as in a normal individual; and on the fourth day, when the parasites were very numerous, the rat was bled for the purpose of another experiment.

Experiment 2. The liquid contents of a tube, inoculated from mouse No. 16 and kept at room temperature for fourteen days, was injected into the peritoneal cavity of a white mouse, and, although some motile trypanosomes were present in the fluid, no infection followed. On October 14, forty-eight days later, it was reinoculated with Culture A, Generation IV*a*. The trypanosomes appeared in the blood a day or two later than in the controls (Nos. 9 and 10, which see), and death followed in eight and one-half days; the two controls dying in seven and seven and one-half days respectively. This would apparently indicate that some slight protection was imparted by the previous inoculation. This matter will be referred to again under immunity.

Experiment 3. The blood from a white rat (No. 21), extremely rich in trypanosomes, was inoculated into blood agar tubes on August 24. The contents of one of these tubes showed living parasites on September 2 (ninth day). A white mouse injected with this material died in ten days. At the time the animal was found it had been dead evidently for some hours, since no trypanosomes could be detected in its blood. To make sure that it died of Nagana another mouse was inoculated with its blood, and died of the disease on the seventh day. Inasmuch as the number of living trypanosomes injected into the latter animal was probably less, since no living forms could be recognized, than was used for the first mouse, it follows that the long course of the disease, ten days, is not due to the injection of a few, but rather to markedly attenuated parasites.

Experiment 4. On September 9 the fluid contents of three tubes, kept at room temperature for two, four, and eight days respectively, were injected into three white mice. The tubes had been inoculated with blood rich in trypanosomes. Mice Nos. 1 and 2, which received the two- and four-day-old fluids respectively, died on the fifth day of Nagana. Mouse No. 3, which received the eight-day fluid, containing living trypanosomes, developed no infection. On September 27 it was reinoculated, this time with virulent Nagana blood, and died in four days.

Experiment 5. On October 17 six tubes of blood agar were inoculated with the blood of a rat, drawn during life and rich in parasites. By inoculating a set of tubes with the blood of one animal and testing these at definite intervals, it seemed as if better results could be obtained than with tubes inoculated at different times with blood from different animals as in the preceding experiment. The tubes were placed at 25°. On the third, fifth, eighth, and tenth day the contents from one of these tubes was injected into a white rat. The rat which received the fluid from the tube three days old showed trypanosomes in its blood on the third and died on the seventh day. The rat which received the contents of the five-day tube showed a very few trypanosomes in its blood on the eighth and died on the twelfth day. Motile trypanosomes were present in the tubes on the eighth and also on the tenth day, but the rat inoculated with fluid from the eight-day tube showed no infection, whereas the one which received the ten-day fluid died in seven days. Its spleen was small, and no trypanosomes could be detected in its blood. Nevertheless, a rat inoculated with this blood died in six days, and trypanosomes were found in its blood. Evidently, survivals ten days old may infect. The failure of the eight-day fluid to infect may have been due to a greater resistance of the rat or to some slight variation in the medium which thereby weakened the parasite, more so than did the ten-day fluid.

The above experiments show that the virulence of surviving trypanosomes, in blood agar tubes, may be maintained for a longer period than under ordinary conditions, since infection was obtained on the fifth, and even on the ninth and tenth day. Under the conditions which obtain in the tubes the organisms, however, usually become non-virulent after the fourth or fifth day. The long duration of the disease (ten and twelve days), and the further fact that the older cultures, though containing living trypanosomes, are devoid of virulence, show that a progressive attenuation is taking place.

It is a well-known fact that a blood may be highly virulent without containing any trypanosomes, recognizable as such under the microscope. A single virulent cell, which perhaps may have escaped observation, is therefore able to produce death in a very short time. An entirely different result follows the injection of even large numbers of living trypanosomes which are either, as in the above experiments, survivals, or actual cultures weakened by prolonged exposure to unfavorable conditions. The period of incubation and the duration of the disease is prolonged, or else no infection results. It is evident that these facts are explainable only on the supposition that actual attenuation takes place. We have not as yet been able to obtain a culture which could be said to be attenuated throughout its development in the tube. The cultures, as will be shown presently, are virulent in the early stages of their growth, after which they weaken and even become non-infectious.

Laveran and Mesnil have shown that virulent Nagana blood, when diluted with 50,000 parts of physiological salt solution and injected into the peritoneal cavity of a mouse, causes death in six and one-quarter days. They have also shown that when blood which is very poor in parasites is injected into mice and rats the trypanosomes may not appear in their blood before the fifth to the seventh day. The injection, into mice, of blood which has been kept at room temperature for thirty-six to forty-eight hours is followed by the appearance of the parasites in the blood on about the ninth day. They have noted the same delay in the appearance of the parasites in the blood after the injection of material which had been kept in an ice-box, or which had been heated to 40–43°, or exposed to the action of injurious chemicals. The prolonged period of incubation in these instances is, in view of our own results, due to a rapid attenuation of the trypanosomes. The same explanation may hold true in the case of Bruce's experiments on dogs, in which a prolonged duration of the disease followed the bites of infected flies which had been kept for twelve to forty-eight hours.

INOCULATION OF PURE CULTURES.

In the previous set of experiments the inoculations were made with the surviving trypanosomes as found on blood agar media where apparently no multiplication had taken place. When, however, undoubted cultures were at last obtained, it became necessary to test these on animals; first, with the object of demonstrating that the cultures were really those of *Trypanosoma brucei*; and, second, to confirm the conclusion arrived at from the preceding experiments with reference to the existence of attenuated or non-virulent forms.

It was soon found that the virulence of the trypanosomes was not a fixed quality but that it is easily influenced by altering the external conditions, such as temperature, the duration of the cultivation and possibly the composition of the medium. These facts had already been foreshadowed in the results obtained by inoculation of the survivals.

The inoculation of mice and rats with the pure cultures completed the evidence necessary to show that the organism cultivated was in reality *Trypanosoma brucei*, and, at the same time, supplied the final proof, if any was needed, that this trypanosome was the cause of Nagana. This organism has now been cultivated *in vitro* for one hundred days, through eight generations, and, as will be seen from Table II, possesses a virulence not much inferior to that of trypanosome blood. There can be no doubt that, given the best possible conditions, it can be cultivated in fully as virulent a form as that met with in living blood.

The intraperitoneal injection of virulent cultures causes death in mice and rats usually in seven or eight days, exceptionally in as late as eleven days. When it is remembered that these animals usually die in from three to five days after the injection of virulent blood, it will be seen that the cultures employed were already somewhat weakened. The retarded death is not due to the injection of a small number of trypanosomes, since in all these experiments the cultures may be characterized as extremely rich in actively motile parasites.

The virulence of one culture was such that a rat died in three and one-half days (see Table III). Two mice (Nos. 8 and 4*a*) inoculated with another culture died in three and one-half and five and one-half days respectively. Mouse No. 8 had received seventeen days before an injection of a non-virulent culture, while mouse No. 4*a* served as a control for the last injection. This same virulent culture was injected at the same time into another control mouse (4*b*), and an examination of its blood, three days later, resulted in finding only one trypanosome in the cover-glass preparation examined. Subsequent examinations, made at intervals of two days, failed to show any trypanosomes. This is the only time that we have found the parasite in the blood without fatal infection following. It may be that the single cell found was a survival of the material injected, and was hence incapable of multiplication. In that event this mouse must be credited with possessing a most unusual degree of resistance.

It should be stated, however, that in this experiment four mice were inoculated at the same time and with the same material. Two of these had survived previous inoculations of non-virulent cultures, and the other two served as controls. One vaccinated mouse and one control died; the others remained alive. It would seem as if an interchange took place, and that the two deaths were those of the controls, but this could hardly have occurred, since special care was exercised to prevent such accidents.

In mice and rats, after the injection of trypanosome cultures, the parasites appear in the blood, usually on the fifth day, exceptionally as early as the third and as late as the seventh and even the ninth day. After the trypanosomes once appear in the blood they rapidly multiply, with the single exception noted above, and become exceedingly numerous just before death. In from two to four days after the trypanosomes appeared in the blood the animals died.

The trypanosomes which develop in the blood of an animal after an injection of a pure culture are identical, in every respect, with those met with in Nagana. The highly refracting globules, usually two or three in number, which are seen in the cultivated trypanosome are not met with in the inoculated animals. We are inclined to consided these refracting globules as evidence of unfavorable environment, and hence as an indication of partial attenuation.

The isolation of the trypanosomes from the blood of animals infected with the pure cultures is as difficult a task as in the case of those inoculated directly with Nagana material.

The cultures employed for the following inoculations were rich in actively motile trypanosomes. The injections were made, as usual, into the peritoneal cavity. It will be seen that only the thirteen- and twenty-day cultures produced infection. It would seem as if a room culture, after two or three weeks, gradually became deprived of its virulence. In the case of the first generation, it should be remembered that no appreciable growth takes place until after eighteen or twenty days. The unfavorable conditions with which the trypanosomes are struggling may be looked upon as being responsible for the absence of virulence. The subsequent generations, having accommodated themselves to these conditions, regain, for a time at least, their pathogenic properties.

TABLE I.

Showing Results of Inoculation of Cultures of Trypanosoma Brucei Developed at Room Temperature.

	CULTURE AND GENERATION	BLOOD AGAR	AGE OF CULTURE	DATE OF			TIME OF DEATH
				Inoculation	Appearance of Trypanosomes	Death	
Mouse 1	A I	2:3	34 days	Sept. 30	0	0	Recovered
Mouse 4	D I	1:1[5]	29	Oct. 11	0	0	Recovered
Mouse 5	A II	2:1	23	Oct. 11	0	0	Recovered
Mouse 7	A III	1:1	13	Oct. 11	Oct. 16	Oct. 19	8 days
2d inject'n mouse 8	A III*a*	3:1	20	Oct. 28	Oct. 31	Nov. 1	3½ days
2d inject'n mouse 4	A III*a*	3:1	20	Oct. 28	0	0	Recovered
Control mouse 4*a*..	A III*a*	3:1	20	Oct. 28	Oct. 31	Nov. 3	5½ days
Control mouse 4*b* ..	A III*a*	3:1	20	Oct. 28	Oct. 31[6]	0	Recovered

The cultures employed in the following inoculations were developed in an incubator room at 25°, and were extremely rich. It will be seen that all of these cultures proved fatal with the exception of one. The mouse used for this inoculation had survived a previous inoculation of a room culture (see Table I). It was given a second injection of an eighteen-day culture, presumably not very virulent, in order to test its immunity. Inasmuch as all the fluid in the culture tube was used for this injection, it was not possible to make a control. The survival of the animal may be due, therefore, either to a condition of immunity or to the non-virulence of the culture.

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[5] The medium in this case was laked-blood agar. The blood was partially laked by the addition of sterile distilled water (1:3), the mixture being kept at 37° for several hours.

[6] Only one trypanosome found in preparation on the third day; none after that date.

TABLE II.

Showing Results of Inoculation of Cultures of Trypanosoma Brucei Developed at 25°.

	Culture and Generation	Blood Agar	Age of Culture	Date of			Time of Death
				Inoculation	Appearance of Trypanosomes	Death	
Mouse 3	A III	2:1	7 days	Oct. 5	Oct. 14	Oct. 16	11 days
Rat 1	A III	2:1	7	Oct. 5	....	Oct. 16	11
Control mouse 9	A IV*a*	3:2	9	Oct. 14	Oct. 19	Oct. 22	7½
Control mouse 10	A IV*a*	3:2	9	Oct. 14	Oct. 19	Oct. 21	7
2d inject'n mouse 1	A IV*a*	3:2	9	Oct. 14	Oct. 21	Oct. 23	9
2d inject'n mouse 2	A IV*a*	3:2	9	Oct. 14	Oct. 19	Oct. 24	9½
2d inject'n mouse 16	A IV*a*	3:2	9	Oct. 14	Oct. 21	Oct. 23	8½
Mouse 11	A V*a* flask	3:2	6	Oct. 21	Oct. 26	Oct. 29	7½
Rat 2	A V*a* flask	3:2	6	Oct. 21	Oct. 28	Nov. 2	11½
Mouse 6	C II	1:1	12	Oct. 11	Oct. 16	Oct. 19	8
2d inject'n mouse 5	A IV	1:1	18	Oct. 23	0	0	Recovered
Rat 5	A V*b* flask	1:1	10	Nov. 4	....	Nov. 8, A.M.	3½ days[7]
Rat 9	A IV*b* flask	3:2	22	Nov. 6	....	Nov. 9	3 days[8]
Rat 10	A IV*b* flask	3:2	22	Nov. 6	Nov. 13	Nov. 16	10
Control mouse 16	A V flask	1:1	18	Nov. 12	Nov. 18	Nov. 21	9
Control mouse 17	A V flask	1:1	18	Nov. 12	Nov. 20	Nov. 22	10
3d inject'n mouse 4	A V flask	1:1	18	Nov. 12	Nov. 18	Nov. 21	9
2d inject'n mouse 4*b*	A V flask	1:1	18	Nov. 12	Nov. 18	Nov. 20	8

It is very probable that the cultures when kept for three or four weeks at 25° will lose their virulence, but on this point we have as yet no definite data.

TABLE III.

Showing Results of Inoculation of Cultures of Trypanosoma Brucei Developed or Exposed at 34°.

	Culture and Generation	Blood Agar	Age of Culture	Date of			Time of Death
				Inoculation	Appearance of Trypanosomes	Death	
Mouse 2	A III	2:1	1 d. 25°; 3 d. 34°	Oct. 2	0	0	Recov.
Mouse 8	A IV	2:1	9 d. 34°	Oct. 11	0	0	Recov.
Rat 3	A V*a* flask	3:2	6 d. 25°; 5 d. 34°	Oct. 26	0	Nov. 9	14 d.[9]
Rat 4	A V*a* flask	3:2	6 d. 25°; 5 d. 34°	Oct. 26	0	Nov. 9 A.M.	13½ d.[9]
Control rat 5	A V*b* flask	1:1	10 d. 25°	Nov. 4	....	Nov. 8 A.M.	3½ d.
Rat 6	A V*b* flask	1:1	10 d. 25°; 1 d. 34°	Nov. 5	Nov. 11	Nov. 14	9 d.
Rat 7	A V*b* flask	1:1	10 d. 25°; 2 d. 34°	Nov. 6	0	0	Recov.
3d injection rat 8	A V*b* flask	1:1	10 d. 25°; 3 d. 34°	Nov. 7	0	0	Recov.
Mouse 5	A V*b* flask	1:1	10 d. 25°; 3 d. 34°	Nov. 7	0	0	Recov.
Mouse 12	A V*b* flask	1:1	10 d. 25°; 4 d. 34°	Nov. 8	0	0	Recov.
Mouse 13	A V*b* flask	1:1	10 d. 25°; 5 d. 34°	Nov. 9	0	0	Recov.
Mouse 14	A V*b* flask	1:1	10 d. 25°; 6 d. 34°	Nov. 10	0	0	Recov.
Mouse 15	A V*b* flask	1:1	10 d. 25°; 6 d. 34°	Nov. 10	0	0	Recov.

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[7] This served as a control for set of inoculations made with the same material heated at 34°. See Table III.

[8] No trypanosomes could be found in the blood. Moreover, the spleen was small. To check this result the blood of this animal was injected into a mouse. No infection followed, and for that reason the death of Rat 9 cannot be ascribed to Nagana.

[9] See below.

Notwithstanding that the cultures develop more rapidly at 34° than at lower temperature, it is evident from the above table that they are non-virulent. The cultures used for Mice 2 and 8 were actively motile. Such cultures developed from the start at 34° rapidly reach their maximum growth, after which they die out.

The culture used for Rats 3 and 4 was first developed at 25°, after which it was placed at 34°. When examined at the end of five days, only round bodies were present, and there was an entire absence of motile forms. These animals therefore really received injections of dead cultures. Although their blood was repeatedly examined, no trypanosomes could be detected. Both rats died on the fourteenth day. The spleen was small, and no trypanosomes could be found in the blood. A mouse was inoculated with the blood of one of these rats and developed no infection.

The death of these animals, unless it be a mere accident, can be explained only on the supposition that the large amount of dead trypanosomes injected caused a fatal intoxication.

In order to test the action of a temperature of 34° upon cultures first fully developed at a lower temperature, a flask culture grown at 25° for ten days was selected, and after injecting a rat (No. 5) with a portion of the fluid, for the purpose of a control, it was set aside at 34°. On each of the following days a portion of the fluid was removed and injected into rats. The result of this experiment is shown in Table III. The flask, after being kept at 34° for three days, began to show involution forms. Moreover, the fat-like globules within the trypanosomes increased in number and size. Motile trypanosomes were still present, though few in number, on the fifth day. No motile forms and only degenerations were present on the sixth day. Two drops of the fluid were injected into a mouse (No. 14), and the remainder of the fluid about $\frac{3}{4}$ c.c., was injected into another mouse (No. 15).

It will be seen from the table that the control rat, No. 5, died in three and one-half days after inoculation. Its spleen was somewhat enlarged. The rat had been dead for some hours, and an injection of a suspension of the internal organs failed to infect a mouse. The trypanosomes had clearly disappeared from the cadaver. The same material, though very rich in motile trypanosomes, after an exposure of twenty-four hours at 34° gave a retarded infection, seen in the fact that the parasites did not appear in the blood until on the sixth day, and death did not occur until the ninth day. The other animals of the series showed no infection.

This experiment shows that an exposure of less than forty-eight hours at 34° is sufficient to destroy the virulence of the cultivated trypanosome, which, however, may remain alive and very motile at this temperature for about five days.

The results of another experiment go to show that, while the 25° culture is attenuated by an exposure of two days at 34° with reference to the new rat, it is still sufficiently virulent to kill rats whose resistance had been lowered by previous inoculation.

Inasmuch as the trypanosomes grow normally in the bodies of animals at a temperature of 37 to 39°, and, under such conditions, maintain their virulence indefinitely, it follows that the temperature is not the only factor which weakens the trypanosome cultures. It has been shown that in whole blood, or even in blood diluted with salt, or citrate solution, or with serum, the trypanosomes die out in anywhere from a few hours to five or six days. The death of the trypanosomes is not due to their inability to multiply outside of the body, but rather to the presence, in more or less concentrated form, of injurious substances probably formed as a result of the death changes in the composition of the blood. In the blood agar medium the injurious action of these products may be considered to be largely neutralized by the agar constituents. That this neutralizing or antitoxic action is not complete is seen in the fact that the room cultures lose their virulence as they age. This effect is more accentuated and is hastened in cultures developed at a higher temperature. As yet we know nothing about the nature of these toxic or antitoxic substances. We are, however, of the opinion that toxic salts or ions are responsible for the rapid destruction of the trypanosomes in dead blood, and, if such be the case, it will be possible to obviate their action. Experiments along this line are now being conducted.

IMMUNITY.

The existence of living and non-virulent cultures of *Trypanosoma brucei* indicates the possibility of immunizing animals by means of such cultures. Thus far no means of protection has been found against the ravages of Nagana, nor for the equally important diseases Surra and Caderas. Even experimentally it has not been possible to protect susceptible animals against infection. Sheep, goats, and cattle are less susceptible, and recovery from the disease protects against subsequent inoculation.

Koch suggested the possibility of attenuating the Nagana trypanosomes by repeated passage through different species of animals, and Schilling endeavored to apply this method, but his results may be said to be highly unsatisfactory, and it is very doubtful if any real protection is obtainable by such procedure.

We have already shown that the Nagana trypanosome can be attenuated by cultivation *in vitro*, and especially by exposure of such cultures to a temperature of 34°. Whether such cultures are able to impart to animals a sufficient degree of immunity so as to protect them against inoculation with virulent blood, we are as yet unable to state. Our own experiments along this line are as yet too few to justify any positive statement. It is our intention to follow out this line of inquiry, and such results as may be obtained will form the subject of a special article.

The result of one experiment of this kind has already been alluded to and is summarized in Table I. It will be seen that the control and treated animals behaved alike; that is to say, one of each died and one of each survived. The control which survived showed a single trypanosome in a cover-glass preparation on the third day, but none thereafter. The treated mouse, No. 4, which recovered, at no time showed the parasites in its blood. The contradictory results of this experiment suggest that an interchange of the animals had in some way taken place, and that the surviving mice were those which had been treated. In view of the great care which was taken to prevent such interchange, it hardly seems probable that this could have occurred. It should perhaps be stated that each of the animals received $\frac{1}{4}$ c.c. of a very rich culture, and that all four injections were made from one charge of the syringe. Other experiments now under way will help to give a definite solution in this problem.

Another immunity experiment is given in Table II. Two control mice, Nos. 9 and 10, and three treated mice, Nos. 1, 2, and 16, were injected with the same culture. It will be noted that the controls died in seven and seven and one-half days, while the treated mice died in eight and one-half, nine, and nine and one-half days. The single treatment to which these mice were subjected apparently gave some slight protection. The failure to secure perfect immunity may be ascribed to the fact that the material injected in the first inoculation was too feeble. Thus mouse No. 1 (see Table I) received a first generation, thirty-four days old. Mouse No. 2 (Table III) received an obviously feeble culture, since it was developed only for one day at 25°, and then for three days at 34°. Mouse No. 16 received on August 27 an injection of the fluid of a tube, inoculated fourteen days before with the blood of a Nagana rat, and containing only surviving trypanosomes.

It is quite likely that mouse No. 5, as shown in Table II, had acquired considerable protection against a very rich and probably virulent culture as the result of a previous injection of a twenty-three-day room culture (Table I). Repeated injections of attenuated cultures will undoubtedly be necessary in order to secure immunity to virulent cultures and to Nagana blood.

MORPHOLOGY.

The appearance of *Trypanosoma brucei*, in cultures, is extremely characteristic and is wholly unlike that of *Tr. lewisi*. When the culture fluid containing the latter organism is examined under a cover-glass, the individual cells will be found to be clear and almost homogeneous. *Trypanosoma brucei*, on the other hand, invariably shows two large, highly refracting globules at the anterior or flagellated end. Occasionally a third and smaller granule is found toward the posterior end. The globules will be found to increase in number and in size when a fully developed culture, grown at room temperature, or at 25°, is placed at 34° for several days. In such a case the globules may measure 0.8 and even 1μ in diameter. A single cell may contain five or six of the large bodies, besides many very small granules. These globules are slightly greenish in color, and when preparations are treated with osmic acid, for twenty-four to forty-eight hours, they darken perceptibly, though at no time do they become perfectly black. It would seem as if these bodies consisted largely of fat, though the presence of paramylum, as in other flagellates, is possible.

The presence of these globules in cultures and their entire absence in the trypanosomes found in the animal body would seem to indicate an alteration in the functional activity of the cells. The fact that the globules are increased in number and in size when the culture is exposed to 34°, in view of the fact that such exposure rapidly destroyed the virulence of the organisms, certainly goes to support this conclusion.

It is probable that, with an improvement in the culture medium, trypanosomes will be obtained free from globules, in which case they will correspond to or resemble exactly those found in the animal. As yet, however, we have not been able to obtain a single culture which did not contain these bodies.

The *Tr. lewisi* as found in cultures varies greatly in size. It is not unusual to find minute forms which, not counting the whip, are but 1 or 2μ in length. There are others which are typical in form which are not much longer than the diameter of a red blood cell. While most of the spindle-shaped cells range from 15 to 20μ in length, some trypanosomes can be found, at times, which are 50 to 60μ long. The existence of the small form accounts for the fact that we have repeatedly been able to infect rats with Berkefeld filtrates of such cultures.

Trypanosoma brucei, on the other hand, shows much less variation in size. The living cell usually measures about 15 to 17μ in length. The *Tr. brucei* is longer and proportionately narrower than the *Tr. lewisi*. The flagellum in the living cell is by no means as distinct and as long as that of the latter organism.

The motion of the two trypanosomes is entirely different. Free swimming or "swarming" forms are common in the case of *Tr. lewisi.* By means of the long flagellum this organism is capable of moving about with great rapidity and in an almost straight line. The motion of *Tr. brucei* is slow and wriggling, and only exceptionally is a slowly progressive form observed. The wave motion slowly passes along the thick, undulating membrane, and gives the appearance of a spiral rotation to the entire cell.

The most striking difference is seen in the appearance of the colonies or aggregates. In the case of *Tr. lewisi* the individual cells are usually grouped in rosette-like aggregations or colonies. These colonies may consist of a dozen or so, or even of hundreds of cells. Again, a number of these colonies may partially coalesce and give rise to enormous multiple colonies which may consist of many thousands of individuals. The colonies present an orderly, symmetrical, and beautiful appearance. The individual cells composing a rosette are spindle-shaped and have a more or less active swaying motion. The flagella in such a colony are not recognizable on the periphery, as in the case of agglutination rosettes. Such rosettes stained by Romanowsky's method show the centrosome close to and below the nucleus; that is to say, the centrosome is nearer the center of the rosette than is the nucleus. This would seem to indicate that the peripheral end is the anterior end, and that the flagella develop there when the cultural conditions are most nearly perfect. This would seem to be the case in the small multiplication rosettes observed in the living blood of the rat.

On the other hand, the cultures of *Tr. brucei* will usually show many cells, in pairs, united by their posterior ends and corresponding to the so-called conjugation forms of Bradford and Plimmer. Larger masses or colonies consisting of ten to twenty cells are met with. The individuals composing these colonies have not the rigid spindle form of *Tr. lewisi*, but are long, narrow, and writhing, and the effect of the whole mass is very suggestive of the snakes on a Medusa head. The flagella appear to be directed outward.

The mode of multiplication of *Tr. brucei* and of *Tr. lewisi* need not be considered at present, inasmuch as this matter will be treated in a subsequent paper.

SUMMARY.

The results of this investigation may be briefly summarized as follows:

1. *Trypanosoma brucei* is more sensitive and more exacting in its requirements than is *Tr. lewisi.* It may, however, be cultivated, *in vitro*, in tubes, and in flasks, by applying the same method which was used for obtaining pure cultures of the rat trypanosome.
2. The chief difficulty consists in obtaining the first or initial culture. Of the first fifty animals from which cultures were attempted only four gave positive results. One of these cultures has been carried on continuously from August 27 up to December 5 (one hundred days), and in this time has been passed through eight generations. There is no reason why the culture should not be maintained indefinitely.
3. It is possible to separate *Tr. lewisi* and *Tr. brucei* when these are present simultaneously in the blood of an animal, by artificial cultivation, since the former will grow on a medium containing one-half or less of its volume of blood, whereas *Tr. brucei* will not grow under such conditions.
4. The cultural characteristics are such as to enable perfect differentiation between the two trypanosomes.
5. The actively growing cultures of *Tr. brucei* possess a virulence which is very nearly the same as that of the original Nagana blood. As the cultures age, and especially by exposure to a temperature of 34°, they become less virulent and eventually, though living, may fail to infect animals.
6. Repeated injections of the attenuated cultures will probably impart solid immunity. In this way it may be possible to secure protection against the ravages of Nagana.
7. The pathogenic action of the pure cultures of *Tr. brucei* demonstrates the causal relationship which this organism bears to Nagana.

8. It may be considered as certain that the method which has proved successful for *Tr. lewisi* and *Tr. brucei* will prove equally useful for other trypanosomes. It will be feasible to subject the trypanosomatic diseases to experimental studies which heretofore have been impossible. The results of these studies may lead to a clearer insight into the causation of protozoal diseases. It seems as if the time is not far distant when most of the pathogenic protozoa will be cultivated and studied in our laboratories in the same way as is now done with bacteria.